

# **EXHIBIT 25**

**Amendment in Response to  
November 3, 2008 Office Action**

Submitted: May 4, 2009

**Serial No. 10/821,726**

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.



## RNAi in *C. elegans*: Soaking in the Genome Sequence

Hiroaki Tabara, Alla Grishok, and Craig C. Mello\*

The completion of the *Caenorhabditis elegans* genome sequence represents a major milestone in a journey initiated by Sydney Brenner some 30 years ago. The goal then as now was to discover how genetic information specifies the development, anatomy, and behavior of a simple animal. Bringing the full potential of the genome sequence to bear on this goal will require facile new reverse genetic tools for converting sequence information into functional information. Here, we briefly describe progress toward understanding and using one such tool termed "RNA interference" or "RNAi."

RNA interference was discovered by Guo and Kemphues (1) in the course of attempts to use antisense RNA to block gene expression in the maternal germ line. To their surprise, they found that both antisense and sense RNA preparations induced remarkably precise phenocopies of the targeted gene. Since then, both the efficacy and apparent lack of strand specificity associated with this interference process have been borne out in many subsequent studies. The mystery surrounding the mechanism of interference was recently deepened with the discovery that double-stranded RNA (dsRNA) is at least an order of magnitude more potent at inducing interference than are preparations of either single strand (2). The surprising properties of this interference mechanism prompted users to abandon the term "antisense" and to begin referring to the process merely as "RNA interference" (3). The robust nature of the interference effect and the high degree of specificity have allowed RNAi to gain wide acceptance as a reverse genetic tool.

Before we discuss the mechanism of in-

terference, two other remarkable features of RNAi deserve comment. First is the observation that the interfering activity can be transported across cell boundaries. Studies

with dilute RNAs suggest that the ideal target tissue for injections is the intestine, even when the gene of interest is expressed in another tissue such as

the germ line or muscle (Fig. 1). Indeed, Lisa Timmons and Andrew Fire (4) have recently shown that feeding the worms *Escherichia coli* expressing the target gene dsRNA is sufficient to induce some interference. Thus, RNA uptake in the gut and distribution from the intestine to the somatic tissues and germ line can occur. Second, the RNAi effect is remarkably long lived. Potent interference is routinely observed not only in the injected animal but also in all of the injected animal's progeny. Thus, interference can be inherited and can last for as much as

that the injected material, or some derivative of it, interacts directly with the target gene, perhaps silencing transcription at the locus. The alternative is that interference may prevent the processing or translation of the endogenous transcript. Several observations are most consistent with interference at a posttranscriptional step. First, only the sequences present in the mature transcript appear to be effective at inducing interference. Promoter and intron sequences appear to be entirely ineffective (2). Second, some *C. elegans* genes exist in operons that are spliced from a single transcript. If RNAi blocks transcription, then interfering with the 5' cistron would be expected to cause a polar effect that blocks the activity of all downstream cistrons. This does not appear to be the case. Several multicistronic genes have been analyzed (6–8), and in all cases, interfering with the 5' cistrons can be accomplished without disturbing the expression of the downstream cistrons.

Experiments with several maternal mRNAs suggest that RNAi does not destabilize or block the translation of the mature message. After RNAi injection into an adult hermaphrodite, we found that the first postinjection segment of the brood includes individuals that received both a functional maternal mRNA and the interfering RNA (5). In such cases, the zygote carries sufficient functional mRNA to permit zygotic development, but also sufficient interfering

RNA to block gene expression during oogenesis in the next generation. Thus, the prevailing evidence supports interference at some posttranscriptional processing step before formation of the mature mRNA.

In most genes, any RNA segment of about 200 to 1000 nucleotides or greater appears to be capable of inducing interference. However, we and others identified specific gene segments that do not seem to be effective at inducing interference (9, 10). So, it is prudent to try RNAs from several segments of a gene when attempting to induce interference. RNA prepared from genomic DNA inclusive of intron sequence is effective as long as an exon sequence is also present. However, double- or single-stranded DNA appears to be completely ineffective. Whatever the mechanism, it is clear that the steady-state levels of the mRNA product of the target gene are substantially reduced within a few hours after the injection of RNA (2). It is also clear that direct mutagenesis of the target gene, as proposed in a previous review (11), is not likely to be part of the interference mechanism. Base substitutions at the target gene would be easily de-

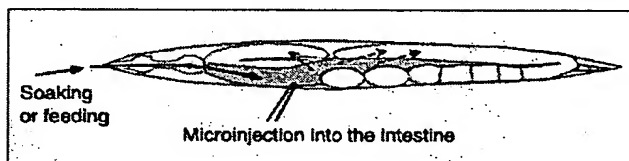


Fig. 1. Delivering RNA to *C. elegans*. The most effective method for blocking gene function is direct microinjection into the cytoplasm of the intestine (orange organ). Feeding and soaking worms holds promise for large-scale applications of RNAi.

several days after the initial injection of RNA. For many genes, interference can persist for at least one full generation after the one receiving the injection, and for certain genes, interference can be observed to transmit in the germ line apparently indefinitely (5). The remarkable ability of the interference effect to cross tissue and cellular boundaries, and the persistence of the effect over a period of several days, together imply the existence of active systems in the worm that mediate RNAi and presumably have some other natural function. The potency and duration of the interference effect also suggests the existence of a catalytic or amplification step in the interference mechanism.

Two models could explain RNAi. One is

The authors are at the Cancer Center, Department of Cell Biology, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605, USA.

\*To whom correspondence should be addressed. E-mail: Craig.Mello@ummed.edu

# SCIENCE'S COMPASS

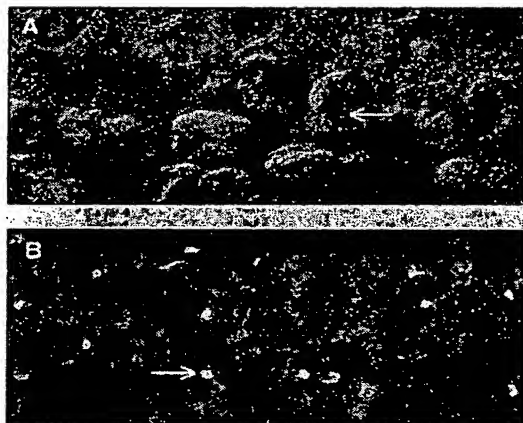
ected and have not been observed to date. Nevertheless, it is conceivable that RNAi is mildly mutagenic, and further investigation will be needed to test this point.

Certain considerations should be kept in mind when interpreting RNAi phenotypes. Whereas in many cases, the RNAi phenotype is very specific and appears similar to that of null alleles for the targeted locus, in other cases, the phenotype may be more similar to that of a weak allele or may only block gene function in a subset of the cells where the target gene is normally expressed. For example, it is not unusual to see strong suppression of a gene in one tissue but little or no suppression of the same gene in another tissue (2, 12). Because it is not yet possible to predict which genes or which tissues will be resistant to RNAi, a lack of phenotype should be followed up with antibody or in situ staining to ensure that expression of the targeted gene is indeed blocked by RNAi. Another worry with RNAi is that RNA targeted for one gene may "cross-interfere" with other closely related genes. Thus the phenotype observed might reflect interference with a different gene or a combination of two or more related genes. This is a valid concern and has been observed on more than one occasion (2, 13). Fortunately, the complete genome sequence provides a nearly perfect set of controls with which to address this issue of cross-interference. First, one can use the genome sequence to identify RNA segments that are unique to a gene of interest. Second, RNA can be prepared from each related gene and injected separately. If two or more related genes give identical RNAi phenotypes, then cross-interference may be occurring, and it is possible that only one of the genes is required for the function of interest. In principle, cross-interference is only a problem when it occurs inadvertently; therefore, RNAi with all closely related genes should be viewed as a standard control for RNAi experiments. In addition, as it is possible to obtain double, triple and possibly higher multiple mutant phenocopies by mixing RNAs (3), it is straightforward to test related genes for redundant or overlapping functions by co-RNAi injections. The maximum number of different genes that can be targeted by a pool of RNA species is not known.

And controlled studies to determine the minimum length and the minimum sequence similarity to induce interference have yet to be reported and are likely to vary for different genes.

The prospects for RNAi are foreshadowed by breakthroughs in the delivery of interfering RNA. Worms fed *E. coli* engineered to express dsRNA from a worm gene

ic RNAi phenotype, upstream and downstream genes could be obtained that function along with the target gene. Furthermore, a similar genetic approach might also be expected to identify general factors required for the interference mechanism itself. One can contemplate mutant strains with an enhanced sensitivity to RNAi, which could be useful as recipient strains for future RNAi experiments. The identification of the genes that mediate RNAi should greatly improve understanding of this mechanism and thus may lead to insights into how best to induce interference. It is possible that these studies will teach us how to transplant RNAi or to activate possibly related mechanisms in other organisms [see (16) for a review]. It will also be interesting to see how far evolution has gone in exploiting this phenomenon. Are there RNA hormones that modulate gene expression in animals? Do cells fight infections by using RNAi to shut down viral genes, or conversely, do pathogens modify the host cell by capturing and overexpressing specific host gene segments? RNAi indicates the existence of a powerful and specific avenue through which RNA from outside the cell can manipulate gene expression on the inside. The possible natural and technical applications are staggering.



**Fig. 2.** (A) Nomarski image showing embryos produced by a wild-type mother treated with *pos-1* RNAi by soaking (14). All except one embryo (arrow) show the distinctive *pos-1* embryonic arrest with no gut, no body morphogenesis, and extra hypodermal cells (15). (B) A lower magnification view showing 137 embryos from the same experiment visualized with polarized light to reveal the differentiation of the birefringent gut granules (arrow). About 14% of the embryos were positive for gut differentiation compared with 1% observed in the *pos-1* null mutant zu148 and 100% observed in the wild type.

can exhibit specific interference with the activity of the targeted gene (14). Simply soaking the worms in dsRNA can also induce specific interference. For example after soaking wild-type worms for 24 hours in dsRNA prepared from the essential maternal gene, *pos-1*, 86% of the  $F_1$  progeny of the soaked animals exhibited the distinctive *pos-1* embryonic lethal phenotype (Fig. 2). Both soaking and feeding appear to work with similar efficiency, but in all cases the effects are less potent than those obtained by direct microinjection (15). In addition, new forms of in vivo, promoter-driven RNAi are likely to provide powerful tools for suppressing gene function. Transgenes that express both strands of RNA and promoters that drive RNA expression in cells other than the target cells (for example, the intestine) may prove to be highly effective at inducing interference.

The ability to induce RNAi en masse may provide an entry point for a variety of genetic studies. For example, by identifying mutations that enhance or suppress a specif-

## References and Notes

1. S. Guo and K. Kemphues, *Cell* **81**, 611 (1995).
2. A. Fire *et al.*, *Nature* **391**, 806 (1998).
3. C. Rochelleau *et al.*, *Cell* **90**, 707 (1997).
4. L. Timmons and A. Fire, *Nature*, in press.
5. A. Grishok and C. C. Mello, unpublished results. We do not know whether this long-term transmission, which is always somewhat less penetrant than the interference observed in the first two generations, involves the same or a secondary interference mechanism.
6. I. Korf, T. Fan, S. Strome, *Development* **125**, 2469 (1998).
7. J. Boshier and M. Labouesse, personal communication.
8. M. Montgomery and A. Fire, personal communication.
9. J. A. Powell-Coffman, J. Knight, W. B. Wood, *Dev. Biol.* **178**, 472 (1996).
10. H. Tabara and C. C. Mello, unpublished results.
11. R. W. Wagner and L. Sun, *Nature* **391**, 744 (1998).
12. C. C. Mello, unpublished observations.
13. In one case, we found that two genes that were 80% identical showed clear evidence of cross-interference. Specific interference was achieved by diluting the injected RNA from 1 mg/ml to 100  $\mu$ g/ml (12).
14. L4-stage hermaphrodites were washed in 0.2 M sucrose and 0.1 M phosphate-buffered saline, and transferred into 10  $\mu$ l of the same buffer in a siliconized tube. In another siliconized tube, 4  $\mu$ l of dsRNA (3.8 mg/ml) and 1  $\mu$ l liposome (Lipofectin, Gibco-BRL) were vigorously mixed. Fifteen worms were added to the RNA-liposome mixture, resulting in a total volume of 15  $\mu$ l and a final RNA concentration of 1 mg/ml. After 24 hours, the worms were transferred to an agar plate with *E. coli* and cultured until mid-adulthood. Similar levels of interference were obtained by feeding worms *E. coli* expressing both strands of *pos-1* RNA (10).
15. H. Tabara and C. C. Mello, unpublished observations.
16. M. K. Montgomery and A. Fire, *Trends Genet.* **14**, 255 (1998).
17. We thank J. Boshier, M. Labouesse, L. Timmons and A. Fire for communicating unpublished results. Work in this laboratory is supported by NIH grant R01 HD33769-01, a pilot and feasibility project grant DK32520-15, and a Pew scholarship to C.C.M.